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(54) Title: METHODS OF PROLONGED SUPPRESSION OF HUMORAL IMMUNITY (57) Abstract <p>Methods of suppressing a humoral immune response to a thymus-dependent (TD) antigen are disclosed. The methods involve administering to a subject a TD antigen with an antagonist of a molecule which mediates contact-dependent helper effector functions. In a preferred embodiment, the antagonist is an antagonist of gp39. Primary and secondary humoral immune responses can be suppressed and suppression is prolonged.</p>			

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METHODS OF PROLONGED SUPPRESSION
OF HUMORAL IMMUNITY

Background of the Invention

5 The immune system is capable of producing two types of antigen-specific responses to foreign antigen. Cell-mediated immunity is the term used to refer to effector functions of the immune system mediated by T lymphocytes. Humoral immunity is the term used to refer to production of antigen-specific antibodies by B lymphocytes. It has long been appreciated that the development of humoral immunity against most antigens requires not only antibody-
10 producing B lymphocytes but also the involvement of helper T (hereafter Th) lymphocytes. Mitchison, *Eur. J. Immunol.*, 1:18-25 (1971); Claman and Chaperon, *Transplant Rev.*, 1:92-119 (1969); Katz et al., *Proc. Natl. Acad. Sci. USA*, 70:2624-2629 (1973); Raff et al., *Nature*, 226:1257-1260 (1970). Certain signals, or "help", are provided by Th cells in response to stimulation by thymus-dependent (hereafter TD) antigens. While some B lymphocyte help is
15 mediated by soluble molecules released by Th cells (for instance lymphokines such as IL-4 and IL-5), activation of B cells also requires a contact-dependent interaction between B cells and Th cells. Hirohata et al., *J. Immunol.*, 140:3736-3744 (1988); Bartlett et al., *J. Immunol.*, 143:1745-1754 (1989). This indicates that B cell activation involves an obligatory interaction between cell surface molecules on B cells and Th cells. Such an interaction is
20 further supported by the observation that isolated plasma membranes of activated T cells can provide helper functions necessary for B cell activation. Brian, *Proc. Natl. Acad. Sci. USA*, 85:564-568 (1988); Hodgkin et al., *J. Immunol.*, 145:2025-2034 (1990); Noelle et al., *J. Immunol.*, 146:1118-1124 (1991).

 A cell surface molecule, CD40, has been identified on immature and mature B
25 lymphocytes which, when crosslinked by antibodies, induces B cell proliferation. Valle et al., *Eur. J. Immunol.*, 19:1463-1467 (1989); Gordon et al., *J. Immunol.*, 140:1425-1430 (1988); Gruber et al., *J. Immunol.*, 142: 4144-4152 (1989). CD40 has been molecularly cloned and characterized. Stamenkovic et al., *EMBO J.*, 8:1403-1410 (1989). A ligand for CD40, gp39 (also called CD40 ligand or CD40L) has also been molecularly cloned and
30 characterized. Armitage et al., *Nature*, 357:80-82 (1992); Lederman et al., *J. Exp. Med.*, 175:1091-1101 (1992); Hollenbaugh et al., *EMBO J.*, 11:4313-4319 (1992). The gp39 protein is expressed on activated, but not resting, CD4⁺ Th cells. Spriggs et al., *J. Exp. Med.*, 176:1543-1550 (1992); Lane et al., *Eur. J. Immunol.*, 22:2573-2578 (1992); Roy et al., *J. Immunol.*, 151:1-14 (1993). Cells transfected with the gp39 gene and expressing the gp39
35 protein on their surface can trigger B cell proliferation and, together with other stimulatory signals, can induce antibody production. Armitage et al., *Nature*, 357:80-82 (1992); Hollenbaugh et al., *EMBO J.*, 11:4313-4319 (1992).

 While the induction of a humoral immune response is an important host defense mechanism, in certain situations it would be beneficial to suppress antibody production

against a particular antigen. For example, suppression of a humoral response against an allergen could prevent or reduce an allergic response in an individual. Additionally, when a therapeutic antibody is administered, suppressing a humoral response against the antibody could prolong the therapeutic efficacy of the antibody.

5

Summary of the Invention

One approach to suppressing humoral immunity is to inhibit B cell activation. The current invention pertains to methods for inhibiting a humoral immune response to a TD antigen *in vivo* by inhibiting the ability of a Th cell to stimulate a B cell, thereby interfering with B cell activation and antibody production. The invention is based, at least in part, on the necessity for an *in vivo* interaction between gp39 on a Th cell and CD40 on a B cell for subsequent activation of the B cell. Antagonists of gp39, which are effective for inhibiting the interaction of gp39 with CD40 *in vivo*, are administered to a subject together with a TD antigen to suppress humoral immunity against the TD antigen. The gp39 antagonist which is administered can be an antibody directed against gp39. In a preferred embodiment, the gp39 antagonist is a monoclonal antibody, such as a anti-human gp39 antibody or an anti-mouse gp39 antibody (e.g., MR1). Chimeric antibodies, humanized antibodies and antibody fragments are also within the scope of the invention. Alternatively, the gp39 antagonist can be a soluble form of the gp39 ligand CD40. Soluble fusion proteins of CD40 are also encompassed by the invention.

The humoral immune response inhibited by the methods of the current invention can be a primary humoral immune response, in the case of initial exposure to an antigen, or a secondary humoral immune response, in the case of reexposure to a previously encountered antigen. For example, the methods described herein can be used to inhibit production of antigen-specific IgM antibodies, IgG antibodies, IgD antibodies and/or IgE antibodies. In addition, the methods provide for prolonged suppression of humoral immune responses *in vivo*.

One aspect of the invention provides methods for inhibiting humoral immune responses to TD antigens. Antigens embraced by the invention include antigens for which specific antibody production requires interaction of gp39 with a ligand on the surface of B cells (e.g., CD40). TD antigens generally include proteinaceous antigens. In preferred embodiments of the invention, the antigen is a therapeutic antibody, drug, allergen or foreign cell. The methods of the present invention also are effective for inhibiting humoral immune responses to a TD antigen while preserving humoral immune responses to thymus-independent type II (hereafter TI-2) antigens.

Another aspect of the invention pertains to methods for specifically inhibiting the helper function of activated Th cells *in vivo* by interfering with the interaction of gp39 with a ligand on the surface of B cells (e.g., CD40) by administering a gp39 antagonist. According

to this method, helper function of activated Th cells is inhibited *in vivo* without deleting or anergizing Th cells.

The invention further pertains to methods of inhibiting humoral immune responses *in vivo* by a combined administration of a gp39 antagonist and another immunosuppressive agent. Other immunosuppressive agents which can be provided in conjunction with a gp39 antagonist include cytokine inhibitors, inhibitors of the CD28/CTLA4 T cell costimulatory pathway, or immunosuppressive drugs.

A still further aspect of the present invention is a method for determining whether an antigen is a TD or TI-2 antigen. This can be determined by whether or not humoral immune responses to the antigen *in vivo* can be inhibited by administration of a gp39 antagonist.

Brief Description of the Drawings

Figure 1A is a bar graph depicting suppression of primary anti-SRBC IgM antibody production by *in vivo* anti-gp39 treatment.

Figure 1B is a graph depicting prolonged suppression of primary anti-SRBC IgM antibody production after short-term *in vivo* anti-gp39 treatment.

Figure 2A is a bar graph depicting suppression of secondary anti-KLH antibody production (various isotypes) by *in vivo* anti-gp39 treatment. Antibody titers were measured 7 days after antigen challenge.

Figure 2B is a bar graph depicting suppression of secondary anti-KLH antibody production (various isotypes) by *in vivo* anti-gp39 treatment. Antibody titers were measured 14 days after antigen challenge.

Figure 3 is two bar graphs depicting suppression of primary anti-ChiL6 IgM antibody production (left) and secondary anti-ChiL6 IgG1 antibody production (right) by *in vivo* anti-gp39 treatment.

Figure 4A is a bar graph depicting suppression of primary anti-TNP IgM antibody production by immunization with TNP-SRBC and *in vivo* anti-gp39 treatment.

Figure 4B is a bar graph depicting lack of suppression of primary anti-TNP IgM antibody production by immunization with TNP-Ficoll and *in vivo* anti-gp39 treatment.

Figure 5 is a bar graph depicting intact helper activity of T cells previously exposed to anti-gp39 treatment *in vivo* upon adoptive transfer to untreated mice, demonstrating that anti-gp39 administration does not functionally delete Th cells.

Figure 6A is a Western blot depicting anti-gp39 antibody present in serum 7, 14 and 21 days after *in vivo* administration.

Figure 6B is a graph depicting percent of remaining anti-gp39 activity in serum 7, 14 and 21 days after *in vivo* administration.

Figures 7A, B and C are flow cytometric profiles depicting the staining of 6 hour activated human peripheral blood lymphocytes with either CD40Ig (panel A), mAb 4D9-8 (panel B) or mAb 4D9-9 (panel C).

Figures 8A, B and C are flow cytometric profiles depicting the staining of 6 hour activated human peripheral blood lymphocytes cultured in the presence of cyclosporin A stained with either mAb 4D9-8 (panel A), mAb 4D9-9 (panel B) or CD40Ig (panel C).

Figures 9A and B are flow cytometric profiles depicting the staining of 6 hour activated human peripheral blood lymphocytes with CD40Ig in the presence of unlabeled mAb 4D9-8 (panel A) or unlabeled mAb 4D9-9 (panel B).

Figure 10 is a graphic representation of the inhibition of human B cell proliferation induced by soluble gp39 and IL-4 when cells are cultured in the presence of anti-human gp39 mAbs 4D9-8, 4D9-9, 24-31, 24-43, 89-76 or 89-79.

Figure 11 is a graphic representation of the inhibition of an allo-specific mixed lymphocyte response when cells are cultured in the presence of anti-human gp39 mAbs 24-31 or 89-79.

Detailed Description of the Invention

The generation of humoral immunity to thymus-dependent (TD) antigens requires not only B lymphocytes, which can produce specific antibodies against an antigen, but also contributions from Th cells which are necessary for activation of B lymphocytes. Although T helper cell function does involve production of cytokines utilized by B lymphocytes, the Th cell requirement for B cell activation cannot be eliminated by providing exogenous cytokines to B cells. Rather, a contact-dependent, cell membrane-mediated interaction between B cells and Th cells is integral to induction of humoral responses. A receptor-ligand pair involved in this interaction, CD40 and gp39, has been identified. CD40 is present on B cells and has the ability to bind to gp39, which is induced on Th cells upon activation, leading to stimulation of the B cells and ultimately production of specific antibodies. Disruption of the CD40-gp39 interaction offers a means of interfering with the generation of a specific humoral immune response.

Accordingly, this invention pertains to methods of inhibiting humoral immune responses against a TD antigen *in vivo*. Humoral immune responses are inhibited by interfering with the interaction of a molecule on a Th cell which mediates contact-dependent helper effector function and its ligand on the surface of a B lymphocyte. In a preferred embodiment, humoral immune responses are inhibited by interfering with the interaction of gp39 on a T cell and CD40 on a B cell exposed to the TD antigen through administration of a gp39 antagonist to a subject *in vivo*. In one embodiment, the B cell is exposed to the TD antigen by administration of the antigen *in vivo* with the gp39 antagonist. Preferably, this TD antigen is a therapeutic agent, for example a therapeutic antibody or drug, which is administered to the patient for therapeutic treatment and for which inhibiting humoral immune responses against it can result in prolonged therapeutic efficacy of the agent. In another embodiment, the TD antigen is an antigen to which a subject is exposed to environmentally, for example an allergen, in which a humoral immune response is

detrimental to the subject, for example results in an allergic reaction. Inhibition of a humoral immune response in this situation would be therapeutically beneficial to the subject.

I. GP39 ANTAGONISTS

5 According to the methods of the invention, a gp39 antagonist is administered to a subject to interfere with the interaction of gp39 on T cells with a gp39 ligand on B cells. A gp39 antagonist is defined as a molecule which interferes with this interaction. The gp39 antagonist can be an antibody directed against gp39 (e.g., a monoclonal antibody against gp39), fragments or derivative of an antibody directed against gp39 (e.g., Fab or F(ab)'2
10 fragments, chimeric antibodies or humanized antibodies), soluble forms of a gp39 ligand (e.g., soluble CD40), soluble forms of a fusion protein of a gp39 ligand (e.g., soluble CD40Ig), or pharmaceutical agents which disrupt the gp39-CD40 interaction.

A. Antibodies

15 A mammal, (e.g., a mouse, hamster, or rabbit) can be immunized with an immunogenic form of gp39 protein or protein fragment (e.g., peptide fragment) which elicits an antibody response in the mammal. A cell which expresses gp39 on its surface can also be used as the immunogen. Alternative immunogens include purified gp39 protein or protein fragments. gp39 can be purified from a gp39-expressing cell by standard purification
20 techniques; gp39 cDNA (Armitage et al., *Nature*, 357:80-82 (1992); Lederman et al., *J. Exp. Med.*, 175:1091-1101 (1992); Hollenbaugh et al., *EMBO J.*, 11:4313-4319 (1992)) can be expressed in a host cell, e.g. bacteria or a mammalian cell line, and gp39 protein purified. gp39 peptides can be synthesized based upon the amino acid sequence of gp39 (Armitage et al., *Nature*, 357:80-82 (1992); Lederman et al., *J. Exp. Med.*, 175:1091-1101 (1992);
25 Hollenbaugh et al., *EMBO J.*, 11:4313-4319 (1992)). Techniques for conferring immunogenicity on a protein include conjugation to carriers or other techniques well known in the art. For example, the protein can be administered in the presence of adjuvant. The progress of immunization can be monitored by detection of antibody titers in plasma or serum. Standard ELISA or other immunoassay can be used with the immunogen as antigen
30 to assess the levels of antibodies.

 Following immunization, antisera can be obtained and, if desired, polyclonal antibodies isolated from the sera. To produce monoclonal antibodies, antibody producing cells (lymphocytes) can be harvested from an immunized animal and fused with myeloma cells by standard somatic cell fusion procedures thus immortalizing these cells and yielding
35 hybridoma cells. Such techniques are well known in the art. For example, the hybridoma technique originally developed by Kohler and Milstein (*Nature* (1975) 256:495-497) as well as other techniques such as the human B-cell hybridoma technique (Kozbar et al., *Immunol. Today* (1983) 4:72), the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al. *Monoclonal Antibodies in Cancer Therapy* (1985) (Allen R. Bliss, Inc., pages 77-

96), and screening of combinatorial antibody libraries (Huse et al., *Science* (1989) 246:1275). Hybridoma cells can be screened immunochemically for production of antibodies specifically reactive with the protein or peptide and monoclonal antibodies isolated.

5 The term antibody as used herein is intended to include fragments thereof which are also specifically reactive with gp39 protein or peptide thereof or gp39 fusion protein. Antibodies can be fragmented using conventional techniques and the fragments screened for utility in the same manner as described above for whole antibodies. For example, F(ab')₂ fragments can be generated by treating antibody with pepsin. The resulting F(ab')₂ fragment can be treated to reduce disulfide bridges to produce Fab' fragments. The antibody of the
10 present invention is further intended to include bispecific and chimeric molecules having an anti-gp39 portion.

When antibodies produced in non-human subjects are used therapeutically in humans, they are recognized to varying degrees as foreign and an immune response may be generated in the patient. One approach for minimizing or eliminating this problem, which is preferable
15 to general immunosuppression, is to produce chimeric antibody derivatives, i.e., antibody molecules that combine a non-human animal variable region and a human constant region. Chimeric antibody molecules can include, for example, the antigen binding domain from an antibody of a mouse, rat, or other species, with human constant regions. A variety of approaches for making chimeric antibodies have been described and can be used to make
20 chimeric antibodies containing the immunoglobulin variable region which recognizes gp39. See, for example, Morrison et al., *Proc. Natl. Acad. Sci. U.S.A.* 81:6851 (1985); Takeda et al., *Nature* 314:452 (1985), Cabilly et al., U.S. Patent No. 4,816,567; Boss et al., U.S. Patent No. 4,816,397; Tanaguchi et al., European Patent Publication EP171496; European Patent Publication 0173494, United Kingdom Patent GB 2177096B. It is expected that such
25 chimeric antibodies would be less immunogenic in a human subject than the corresponding non-chimeric antibody.

For human therapeutic purposes the monoclonal or chimeric antibodies specifically reactive with a gp39 protein or peptide can be further humanized by producing human constant region chimeras, in which parts of the variable regions, especially the conserved
30 framework regions of the antigen-binding domain, are of human origin and only the hypervariable regions are of non-human origin. Such altered immunoglobulin molecules may be made by any of several techniques known in the art, (e.g., Teng et al., *Proc. Natl. Acad. Sci. U.S.A.*, 80:7308-7312 (1983); Kozbor et al., *Immunology Today*, 4:7279 (1983); Olsson et al., *Meth. Enzymol.*, 92:3-16 (1982)), and are preferably made according to the teachings of
35 PCT Publication WO92/06193 or EP 0239400. Humanized antibodies can be commercially produced by, for example, Scotgen Limited, 2 Holly Road, Twickenham, Middlesex, Great Britain.

Another method of generating specific antibodies, or antibody fragments, reactive against a gp39 protein or peptide is to screen expression libraries encoding immunoglobulin

genes, or portions thereof, expressed in bacteria with a gp39 protein or peptide. For example, complete Fab fragments, VH regions and Fv regions can be expressed in bacteria using phage expression libraries. See for example Ward et al., *Nature*, 341: 544-546: (1989); Huse et al., *Science*, 246: 1275-1281 (1989); and McCafferty et al., *Nature*, 348: 552-554 (1990).

- 5 Screening such libraries with, for example, a gp39 peptide can identify immunoglobulin fragments reactive with gp39. Alternatively, the SCID-hu mouse developed by Genpharm can be used to produce antibodies, or fragments thereof.

B. Soluble Ligands for gp39

- 10 Other gp39 antagonists which can be administered to suppress humoral immunity are soluble forms of a gp39 ligand. A monovalent soluble ligand of gp39 can bind gp39, thereby inhibiting the interaction of gp39 with CD40 on B cells. The term soluble indicates that the ligand is not permanently associated with a cell membrane. A soluble gp39 ligand can be prepared by chemical synthesis, or, preferably by recombinant DNA techniques. A preferred
15 soluble gp39 ligand is soluble CD40. Alternatively, soluble gp39 ligand can be in the form of a fusion protein. Such a fusion protein comprises at a least a portion of the gp39 ligand attached to a second molecule. For example, CD40 can be expressed as a fusion protein with immunoglobulin (CD40Ig). In one embodiment, a fusion protein is produced comprising a amino acid residues of an extracellular domain portion of the CD40 joined to amino acid
20 residues of a sequence corresponding to the hinge, CH2 and CH3 regions of C γ 1 to form a CD40Ig fusion protein (see e.g., Linsley et al. (1991) *J. Exp. Med.* 1783:721-730; Capon et al. (1989) *Nature* 337, 525-531; and Capon U.S. 5,116,964). The fusion protein can be produced by chemical synthesis, or, preferably by recombinant DNA techniques based on the cDNA of CD40 (Stamenkovic et al., *EMBO J.*, 8:1403-1410 (1989)).

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II. ANTIGENS AGAINST WHICH HUMORAL IMMUNITY IS SUPPRESSED

- The invention is directed to suppressing humoral immunity against antigens which require contact-dependent helper functions delivered by Th cells. Antigens classically described as thymus-dependent (TD) antigens are encompassed by the invention. The
30 necessity for contact-dependent "help" from Th cells can be due to the necessity for an interaction between gp39 on T cells and CD40 on B cells. As defined by the current invention, the term "TD antigen" is intended to encompass antigens which require a gp39-CD40 interaction between T cells and B cells for induction of a humoral immune response against the antigen. In general, protein antigens are TD antigens. Another form of TD
35 antigen encompassed by the invention is a molecule, referred to as a hapten, linked to a protein. In this case, the protein acts as a carrier for inducing T cell help in order to induce humoral immune responses against the hapten.

The TD antigen of the invention can be administered in soluble form to a subject, e.g., injection of a soluble protein, or the TD antigen can be on the surface of a cell, e.g., a cell-

surface protein. The TD antigen can be administered to a subject with a gp39 antagonist or a subject may be exposed to a TD antigen environmentally, for example an allergen. In preferred embodiments, the TD antigen is an agent administered to a subject for therapeutic purposes. This agent can be, for example, a therapeutic antibody or other form of therapeutic drug which is a TD antigen. Inhibiting a humoral immune response against, for instance, a therapeutic antibody, can prolong its efficacy *in vivo* by preventing clearance of the therapeutic antibody in a subject. Small molecules acting as therapeutic agents can also be target antigens against which a humoral response is suppressed if these molecules (functioning as haptens) are administered with a protein or other carrier that induces T cell helper function to activate B cells; suppressing humoral immunity against these therapeutic agents can likewise prolong their effectiveness.

The methods of the invention provide for suppression of humoral immunity against TD antigens while not affecting responses to thymus-independent type II (TI-2) antigens. TI-2 antigens include polysaccharides and lipids which can non-specifically activate B cells in a polyclonal manner. As defined by the current invention, the term "TI-2 antigen" is intended to encompass all antigens which do not require a gp39-CD40 interaction between T cells and B cells for induction of a humoral immune response against the antigen. The current invention provides a method for identifying whether an antigen is a TD antigen or a TI-2 antigen, as defined in the invention, by determining whether humoral immune responses to the antigen can be inhibited by a gp39 antagonist.

III. SUPPRESSION OF HUMORAL IMMUNITY

The invention pertains to methods of inhibiting a humoral immune response against a TD antigen. The humoral immune response can be a primary immune response, in the case of a first exposure to a TD antigen, or the response can be a secondary humoral immune response, in the case of reexposure to the antigen. Production of one or more isotypes of antibodies can be inhibited. For a primary humoral immune response, in which IgM is the predominant antibody produced, IgM production is predominantly suppressed. For secondary immune response, production of several different isotypes, including IgM, IgG and IgE can be suppressed.

The invention provides methods for prolonged suppression of humoral immunity against a TD antigen. As used herein, "prolonged suppression" means that suppression of antibody production against a TD antigen is maintained after administration of a gp39 antagonist *in vivo* has been terminated.

IV. ADMINISTRATION OF GP39 ANTAGONISTS

Humoral immune responses to a TD antigen can be inhibited according to the methods described herein by administration of a gp39 antagonist to a subject which is exposed to the TD antigen. In one embodiment, the gp39 antagonist is administered in

conjunction with the TD antigen. The gp39 antagonist is preferably administered simultaneously with the TD antigen, but can be administered prior to administering the TD antigen or subsequent to the TD antigen, as long as the gp39 antagonist is administered before the TD antigen has induced B cell activation. In other embodiments, a subject is
5 exposed to an antigen environmentally. In this case, a gp39 antagonist should be administered *in vivo* following exposure to the antigen, in close enough in time to prevent B cell activation.

The antagonists of the invention are administered to subjects in a biologically compatible form suitable for pharmaceutical administration *in vivo* to suppress humoral
10 immune responses. By "biologically compatible form suitable for administration *in vivo*" is meant a form of the antagonist to be administered in which any toxic effects are outweighed by the therapeutic effects of the protein. The term subject is intended to include living organisms in which an immune response can be elicited, e.g., mammals. Examples of subjects include humans, dogs, cats, mice, rats, and transgenic species thereof.
15 Administration of an antagonist which interferes with the interaction of gp39 and CD40 as described herein can be in any pharmacological form and a pharmaceutically acceptable carrier. Administration of a therapeutically active amount of the therapeutic compositions of the present invention is defined as an amount effective, at dosages and for periods of time necessary to achieve the desired result. For example, a therapeutically active amount of an
20 antagonist which interferes with the interaction of gp39 and CD40 may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the antagonist to elicit a desired response in the individual. Dosage regimens may be adjusted to provide the optimum therapeutic response. For example, several divided doses may be administered daily or the dose may be proportionally reduced as indicated by the exigencies
25 of the therapeutic situation.

The active compound (e.g., antagonist) may be administered in a convenient manner such as by injection (subcutaneous, intravenous, etc.), oral administration, inhalation, transdermal application, or rectal administration. Depending on the route of administration, the active compound may be coated in a material to protect the compound from the action of
30 enzymes, acids and other natural conditions which may inactivate the compound.

To administer an antagonist which interferes with the interaction of gp39 and CD40 by other than parenteral administration, it may be necessary to coat the antagonist with, or co-administer the antagonist with, a material to prevent its inactivation. For example, an antagonist can be administered to an individual in an appropriate carrier or diluent, co-
35 administered with enzyme inhibitors or in an appropriate carrier such as liposomes. Pharmaceutically acceptable diluents include saline and aqueous buffer solutions. Enzyme inhibitors include pancreatic trypsin inhibitor, diisopropylfluorophosphate (DEP) and trasylol. Liposomes include water-in-oil-in-water emulsions as well as conventional liposomes (Strejan et al., (1984) *J. Neuroimmunol* 7:27).

The active compound may also be administered parenterally or intraperitoneally. Dispersions can also be prepared in glycerol, liquid polyethylene glycols, and mixtures thereof and in oils. Under ordinary conditions of storage and use, these preparations may contain a preservative to prevent the growth of microorganisms.

5 Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. In all cases, the composition must be sterile and must be fluid to the extent that easy syringability exists. It must be stable under
10 the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the
15 use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, asorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by
20 including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating active compound (e.g., antagonist which interferes with the interaction of gp39 and CD40) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as
25 required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active
30 ingredient (e.g., antagonist) plus any additional desired ingredient from a previously sterile-filtered solution thereof.

When the active compound is suitably protected, as described above, the protein may be orally administered, for example, with an inert diluent or an assimilable edible carrier. As used herein "pharmaceutically acceptable carrier" includes any and all solvents, dispersion
35 media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the therapeutic compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

It is especially advantageous to formulate parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the mammalian subjects to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on (a) the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and (b) the limitations inherent in the art of compounding such an active compound for the treatment of sensitivity in individuals.

V. COADMINISTRATION OF GP39 ANTAGONISTS AND OTHER IMMUNOSUPPRESSIVE AGENTS

It has been shown that the interference by soluble CTLA-4 of CD28 triggering, a co-stimulatory molecule on T_H cells, also suppresses TD antibody responses (30) and blocks xenogeneic graft rejection (31). Similar to anti-gp39 administration, soluble CTLA-4 induced a state of prolonged immune suppression. Because anti-gp39 and CTLA-4 mediate their immunosuppressive effects at distinct stages of the humoral immune response, co-administration of these two immunosuppressive drugs may provide additive or synergistic immunosuppressive effects on immunity.

Allergic responses are mediated by IgE antibodies. The production of IgE responses requires the cytokine IL-4. Inhibition of IgE responses against a TD antigen may be more efficient by coadministration of a gp39 antagonist and an inhibitor of IL-4, for example an anti-IL-4 antibody.

This invention is further illustrated by the following examples which should not be construed as limiting. The contents of all references and published patent applications cited throughout this application are hereby incorporated by reference. The contents of a patent application filed on even date herewith in the name of Randolph J. Noelle et al. and entitled "Methods for Inducing Antigen-Specific T Cell Tolerance" is incorporated herein by reference.

The following methodology was used in the examples.

Materials and Methods

Animals. Female, 6-8 week old Balb/c mice (Jackson Laboratories, Bar Harbor, ME) were used for the *in vivo* experiments presented in this study. Animals were maintained in the specific pathogen-free animal facility at Dartmouth Medical School.

Helper T cell clones (T_H1). D1.6, an I-A^d-restricted, rabbit Ig-specific T_H1 clone (21) was obtained from Dr. David Parker, University of Mass. at Worcester. In this paper, D1.6 will be referred to as T_H1.

- Reagents and Antibodies. MR1, hamster anti-murine gp39 mAb (16) was purified by DEAE HPLC from ascites fluid. Hamster Ig (HIg), used as a control antibody, was purified similarly from hamster serum (Accurate Chemical and Scientific Corp., Westbury, NY).
- 5 RG7/7.6.HL, a mouse anti-rat κ chain (strongly crossreactive with hamster κ chain) antibody, (RG7), (22) was conjugated with HRPO or FITC and used as a secondary reagent to detect MR1 and HIg. Affinity-purified Goat anti-mouse IgM, IgG1, IgG2a, IgG2b and IgG3 (Southern Biotechnology, Birmingham Al) were used as detection antibodies in the antigen specific ELISAs as well as in the total IgM and IgG1 ELISAs. B1E3, (kindly provided by
- 10 Dr. T. Waldschmidt, Univ. of Iowa) a monoclonal anti-murine IgE, was used as the detection antibody for the IgE anti-KLH ELISA. Chimeric-L6 (Chi-L6), a humanized IgG1 specific for the tumor antigen L6 (23), was kindly provided by Bristol-Myers Squibb Pharmaceutical Research Institute, Seattle WA. Anti-CD4, GK 1.5 (24) was prepared by HPLC purification of ascites fluid. Sheep red blood cells (SRBC) were purchased from Colorado Serum Co.
- 15 (Denver, CO). Sea Plaque agarose for use in anti-SRBC plaque assay was obtained from FMC Corporation (Rockland MA). Baby rabbit complement was purchased from Cedarlane (Hornby, Ontario Canada). KLH, Keyhole limpet hemocyanin, (from *Megathura crenulata*) was purchased from Calbiochem (LaJolla, CA). Complete Freund's adjuvant (CFA) for immunizations was obtained from Sigma Chemical Co (St. Louis, MO). TNP-SRBC, TNP-
- 20 KLH and TNP-BSA were prepared as previously described (25).

Immunizations for Generation of In Vivo Primary and Secondary Antibody Responses.

- Primary Immune Responses.* For eliciting primary antibody responses to SRBC or TNP-SRBC, mice were immunized with 200 μ l of 1% SRBC or TNP-SRBC suspension (i.v.). The
- 25 IgM, anti-SRBC response was assayed 5d after administration of antigen using a modification of the Jerne plaque assay (26). IgM anti-TNP responses were measured by ELISA on day 6. Primary responses to the heterologous immunoglobulin Chi-L6 were generated by i.p. immunization of 100 μ g Chi-L6 on alum per mouse. The serum IgM anti-Chi-L6 antibody response was measured after 7d. Primary responses to TNP-Ficoll were
- 30 generated by immunization with 25 μ g of TNP-Ficoll i.p. The IgM anti-TNP response was measured on day 6 by ELISA.

- Secondary Immune Responses.* For generation of secondary humoral responses to KLH, animals were immunized with KLH in CFA (50 μ g; i.p.). Mice were subsequently challenged with 10 μ g of soluble KLH (i.p.) three months later. The anti-KLH antibody
- 35 response was measured on d7 from the serum of immune mice utilizing isotype specific ELISAs. Secondary antibody responses to Chi-L6 were generated by challenging Chi-L6 immune mice with 10 μ g soluble Chi-L6, i.p. The serum IgG1 anti-Chi-L6 antibody response was measured after 7d.

Anti-gp39 Treatment. Sterile, HPLC-purified anti-gp39 (MR1) or HIg (as an antibody control) was administered (i.p.) on d0, d2, d4 post immunization or challenge as indicated for each experiment.

- 5 Antigen Specific ELISAs. The antigen specific IgM, IgG1, IgG2a, IgG2b, IgG3, and IgE antibody titers were determined using isotype specific ELISAs. Briefly, antigen, (1 mg/ml of KLH, Chi-L6, TNP16-BSA, or TNP2-BSA in PBS) was absorbed onto flexible polyvinyl microtiter dishes, overnight at 4°C. Plates were washed and blocked with PBS-1% FCS-sodium-azide. Diluted serum samples were incubated for 2 hours at 37°C. Samples were
10 washed and the antigen specific antibody titers determined with one of the following alkaline-phosphatase conjugated detection antibodies: goat anti-mouse IgM, IgG1, IgG2a, IgG2b, or IgG3 (Southern Biotechnology, Birmingham, Al). The IgE specific ELISA was detected using biotin-conjugated B1E3 followed by alkaline-phosphatase avidin (South San Francisco, CA). All ELISAs were developed by reaction of alkaline-phosphatase with
15 phosphatase substrate (Sigma Chemical, Co., St. Louis, MO). Plates were analyzed on a Dynatech MR700 ELISA reader at 410 nm. Units represent arbitrary values based on the titration curve of a standard immune serum. All experimental groups were titrated from 1:100 to 1:100,000 and the titer ascertained based on multiple point analysis. The levels of anti-KLH, anti-Chi-L6 and anti-TNP antibodies in unchallenged controls were below detection.

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Detection of Serum Anti-gp39.

- Quantitation of intact anti-gp39 in the serum of anti-gp39-treated mice:* Serum from mice receiving 750 µg anti-gp39 (250 µg on d0, d2, d4) was obtained on d7, d14, and d21 after initiation of anti-gp39 treatment. The serum was run on a 7.5% SDS gel under non-reducing
25 conditions, transferred to nitrocellulose, and blotted with HRPO-conjugated RG7. Following chemiluminescent detection, areas of the blot corresponding to 150-165 kDa were scanned and digitized using an *Apple Scanner* and the *Image 4.1* software program.

- Analysis for biologically active anti-gp39 in the serum of treated mice:* Anti-CD3-activated Th1, which express gp39, were stained with dilutions of serum from mice receiving 750 µg
30 anti-gp39 (250 µg on d0, d2, d4) to determine the amount of biologically active gp39 remaining in the serum. Titrations of serum containing anti-gp39 were incubated with activated Th1 cell clones for 30 minutes at 4°C, followed by washing and subsequent incubation with FITC-RG7 for 30 minutes at 4°C. A standard curve of MFI vs anti-gp39 concentration was generated using purified anti-gp39. Samples were analyzed on a Becton
35 Dickinson FACScan and the percent anti-gp39 remaining in the serum was deduced based on the anti-gp39 standard curve. The level of anti-gp39 present in the serum at d7 was set at 100%.

Adoptive Transfer of Helper T cells. Mice were immunized with SRBC (200 μ l of 1% SRBC, i.v.) and administered anti-gp39 or HIg (250 μ g on d0, d2, d4). On d7 the splenocytes from nonimmune or SRBC-immune mice were removed, erythrocyte depleted, washed and transferred (i.v., 50×10^6 /mouse) into irradiated recipients (600 rads) with or without 50×10^6 spleen cells from TNP-KLH primed (TNP-KLH-CFA, 50 μ g i.p.) mice as a source of immune B cells. At the time of transfer, mice were immunized with TNP-SRBC (200 μ l of 1% TNP-SRBC i.v.) Serum IgG1 anti-TNP titers were ascertained on d6 post-transfer.

10 **EXAMPLE 1: Anti-gp39 Inhibits the Generation of Primary**
Antibody Responses to Erythrocyte Antigens

The impaired TD immunity observed in patients with HIM, as well as the potent inhibitory effects of anti-gp39 and CD40-Ig on T_H -dependent B cell activation *in vitro*, provided the basis for the study of the potential immunosuppressive effects of anti-gp39 on humoral-mediated immunity *in vivo*. To investigate the role of gp39-CD40 interactions in primary TD humoral immune responses, the effect of *in vivo* administration of anti-gp39 on the primary antibody response to sheep red blood cells (SRBC) was determined. Animals were immunized with SRBC and administered anti-gp39 mAb (or control HIg) over the course of 4d. On d5, the primary anti-SRBC antibody response of anti-gp39-treated, HIg-treated, and control mice was ascertained. The IgM anti-SRBC plaque-forming cell (PFC) response of mice that received a total of 1.5 mg of anti-gp39 (500 μ g/mouse on d0, d2 and d4) was reduced 99% when compared to the anti-SRBC PFC response from control or HIg-treated mice (Figure 1A). In addition, administration of as little as 300 μ g/mouse (100 μ g/mouse on d0, d2, and d4) of anti-gp39 reduced the anti-SRBC primary immune response by 66%. Results from these experiments demonstrate that anti-gp39 treatment ablates primary antibody responses *in vivo*.

The duration of the immunosuppressive effects of anti-gp39 on the primary humoral immune response to SRBC was subsequently examined. Mice immunized with SRBC were treated with anti-gp39 for 4d and assayed at various later time points for the capacity to mount a primary anti-SRBC response. In this set of experiments, all animals were immunized with SRBC on d0 and administered anti-gp39 or HIg on d0, d2, d4. The IgM anti-SRBC PFC response was measured for one group on d5. Additional SRBC-immune groups were challenged with SRBC on d7 or d14. Five days following the each antigenic challenge (d12 and d19, respectively), the IgM anti-SRBC PFC response was measured. The results of one such experiment are depicted in Figure 1B. As in Figure 1A, the primary anti-SRBC responses were inhibited 80-90% 5d after anti-gp39 administration was begun. In addition, the primary anti-SRBC responses 12d and 19d following anti-gp39 treatment were

also inhibited >90%. These results demonstrate that brief anti-gp39 treatment results in prolonged inhibition of primary antibody responses.

EXAMPLE 2: Anti-gp39 Inhibits the Generation of Secondary Anti-KLH Antibody Responses

Experiments examining primary antibody responses suggest that gp39-CD40 interactions play a critical role in the initiation of primary humoral immunity. However, these experiments do not address the whether gp39-dependent CD40 signalling is required for the generation of secondary antibody responses. Therefore, the effects of anti-gp39 administration on the secondary immune response to soluble challenge with KLH was determined in KLH-immune mice.

Using schedules of anti-gp39 administration that reduced the primary anti-SRBC PFC response, experiments were designed to evaluate the effects of anti-gp39 treatment on the secondary antibody responses. In these experiments, KLH-immune mice (immunized 3 months prior with CFA and KLH) were challenged with soluble KLH (10 µg/mouse/i.v.). On the day of antigen challenge (d0), mice were also given 250 µg of anti-gp39 or HIg, followed by anti-gp39 or HIg on d2 and d4. At d7 (Fig. 2 panel A) and d14 (Fig. 2 panel B) following challenge with KLH, the mice were bled and the titers of IgM, IgG1, IgG2a, IgG2b, IgG3 and IgE anti-KLH antibodies were determined. The results demonstrate several points: 1) challenge with soluble KLH induced an enduring secondary immune response that persisted for up to 14d; 2) the administration of anti-gp39 significantly reduced the secondary anti-KLH response of the isotypes measured when compared to the administration of equal quantities of HIg; and 3) the immunosuppressive effects of anti-gp39 appeared to be sustained for at least 14d after the initiation of anti-gp39 treatment. Taken together, results from these experiments demonstrate that similar to primary humoral immune responses, the generation of secondary humoral immune responses were also blocked by anti-gp39.

EXAMPLE 3: Anti-gp39 Inhibits the Generation of Antibody Responses to Heterologous Ig

Experiments depicted in Figure 1 demonstrate the immunosuppressive activity of anti-gp39 during a primary response to a strongly immunogenic particulate antigen, SRBC. The cellular nature of erythrocytes makes them unique in their capacity to elicit strong immune responses. Heterologous Ig molecules share this characteristic of being highly immunogenic, and therefore provide an additional model antigen system with which to examine the effects of anti-gp39 treatment on the generation of primary and secondary antibody responses. Animals were immunized with a heterologous Ig molecule, Chi-L6, a

humanized mouse anti-tumor cell mAb, and treated with anti-gp39 or control HIg. After 7d, sera was collected and assayed for the production of IgM anti-Chi-L6 antibodies. In addition, mice were challenged with Chi-L6 14d after initial immunization and anti-gp39 treatment, and assayed for IgG₁ anti-Chi-L6 antibody production on d21. Figure 3 depicts the results of one such experiment. The primary antibody response to Chi-L6 in mice treated with anti-gp39 is inhibited by > 90% when compared to HIg-treated mice. Moreover, the secondary, IgG₁ response to Chi-L6 is similarly inhibited. These results demonstrate that anti-gp39 treatment ablates primary and secondary antibody responses to a second type of TD antigen, heterologous Ig, as effectively as it suppresses responses to erythrocyte and soluble protein antigens.

EXAMPLE 4: Anti-gp39 Does Not Inhibit the Generation of Primary Antibody Responses to the T-Independent Type II Antigen, TNP-Ficoll

Although the previous experiments demonstrate that anti-gp39 effectively blocks the generation of primary and secondary antibody responses to TD antigens *in vivo*, it is unclear whether gp39-CD40 interactions play a role in the initiation of humoral responses to TI antigens. Data presented in the accompanying paper demonstrate that immunization with the TI-type II antigen, TNP-Ficoll, results in gp39 expression by T_h cells *in vivo*. In order to address whether gp39-CD40 interactions are necessary for the generation of antibody responses to this TI antigen, the affect of anti-gp39 treatment on mice immunized TNP-Ficoll, was assessed. Mice immunized with TNP-Ficoll or TNP-SRBC were treated with anti-gp39 or HIg and the IgM anti-TNP antibody response determined after 6 days. Figure 4A demonstrates that animals immunized with the TD antigen TNP-SRBC elicit significant anti-TNP serum antibody responses. As predicted from the previously described experiments, anti-gp39 treatment dramatically inhibits the primary anti-TNP response generated in these mice. In contract, mice immunized with TNP-Ficoll mount a higher titered anti-TNP antibody response (Figure 4B); however, treatment with anti-gp39 does not inhibit the antibody response to TNP-Ficoll. Results from these experiments demonstrate that, unlike responses to TD antigens, anti-gp39 does not block the generation of humoral responses to TNP-Ficoll, suggesting that responses to TI antigens may be gp39-independent.

EXAMPLE 5: Anti-gp39 Administration Does Not Functionally Delete SRBC-Specific T_h

From the previous experiments, it is known that anti-gp39 interferes with the development of TD humoral immunity; however, the mechanism by which anti-gp39 treatment suppresses humoral responses is not clear. Immune suppression by anti-gp39

could be mediated by: 1) the negative signalling of gp39-bearing T cells causing T_H anergy; 2) mAb-mediated cytotoxic deletion of anti-gp39 bearing $CD4^+$ T cells; and/or 3) the blocking of gp39 binding to CD40. A series of experiments were performed to gain insight into which of these mechanisms may be operative in the protracted immune suppression observed with anti-gp39 therapy. To explore the possibility that antigen-specific T_H were deleted or anergized by anti-gp39 therapy, antigen-specific T_H function from gp39-treated mice was measured by adoptive transfer. Briefly, mice were immunized with SRBC (to prime SRBC-specific T_H) and administered anti-gp39 or HIg (250 μ g/mouse on d0, d2, d4). After 7d, spleen cells from unimmunized mice or SRBC-immune spleen cells from HIg-treated or anti-gp39-treated mice were adoptively transferred into recipient mice with TNP-immune spleen cells as a source of TNP-primed B cells. Mice were simultaneously challenged with TNP-SRBC, and the IgG1 anti-TNP titer ascertained on d5. SRBC-primed T helper cells are required to elicit a secondary anti-TNP response in the recipient mice as demonstrated by the fact that recipients which received spleen cells from nonimmune donors produced substantially lower IgG1 anti-TNP compared to those mice which received spleen cells from SRBC-primed animals (Figure 5). More importantly, results of these experiments revealed that the SRBC helper activity from HIg-treated and anti-gp39-treated mice was similar, indicating that anti-gp39 treatment did not alter T_H function or block the priming of T_H . Moreover, antigen-responsive T_H were not deleted or anergized as a result of anti-gp39 treatment, as they provided helper-effector function upon transfer.

EXAMPLE 6: *In Vivo* Clearance of Hamster Anti-gp39

Previous studies have established that anti-gp39 (MR1) blocks the binding of gp39 to CD40 (15) and thus support the hypothesis that the *in vivo* immunosuppressive effects of anti-gp39 are due to the blocking of gp39-CD40 interactions. Assuming this hypothesis correct, the long-term immune suppression observed with anti-gp39 administration requires the persistence of anti-gp39 in the host. To determine if anti-gp39 could be detected for the period of time that immune suppression was evident, the *in vivo* clearance rate of anti-gp39 from serum was determined. Mice were given a regime of antibody (3 x 250 μ g anti-gp39) over the course of 4d and assayed for the levels of serum anti-gp39 at 7d, 14d, and 21d after the initiation of antibody administration. Western blot analysis for non-reduced MR1 (160 kd) indicated that intact, serum anti-gp39 could be detected for at least 21d after the initiation of antibody treatment (Figure 6A). The serum concentration of anti-gp39 in animals at 21d was approximately 5% (based on scanning densitometry), when compared to the signals derived from serum of animals analyzed 7d after initiation of antibody therapy.

Although it was determined that intact anti-gp39 was present in serum, it was also important to ascertain that the anti-gp39 was biologically active. Therefore, sera from mice which received 3 x 250 μ g of anti-gp39 over the course of 4d were used to stain gp39-bearing

T_h (Figure 6B). The level of serum anti-gp39 3d after the last injection (7d after initiation of antibody treatment) was set at 100%. Fourteen days after the initiation of antibody therapy, approximately 10-15% of the biologically active anti-gp39 mAb was detected in the serum. Twenty-one days post-initiation of therapy, 2-3% of anti-gp39 remained in the serum.

- 5 Therefore, both the determination of intact gp39 by Western blotting and of biologically active anti-gp39 revealed that approximately 5% of the anti-gp39 was present 21d after beginning anti-gp39 therapy. These results demonstrate the half-life of anti-gp39 to be approximately 12d and offer evidence consistent with the hypothesis that prolonged suppression of humoral immune responses by anti-gp39 is due to persistent blocking of T_h function.
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The present study demonstrates that *in vivo* administration of an anti-gp39 antibody which blocks gp39-CD40 interactions *in vitro*, results in profound inhibition of both primary and secondary humoral immune responses to TD antigens, but not TI-type II antigens. In addition, this study demonstrates that anti-gp39 treatment does not block the priming of antigen-primed T_h cells. Therefore, the gp39-CD40 ligand-receptor pair can be used as a target for the therapeutic manipulation of the humoral immune response.

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To gain insight into how anti-gp39 was exerting its immunosuppressive effect on humoral immunity, the direct effects of anti-gp39 on T_h function were addressed. The data indicate that SRBC-immune T_h from anti-gp39-treated mice were fully capable of providing help upon adoptive transfer, suggesting that anti-gp39 treatment did not cause T_h deletion or anergy *in vivo*. These results led to the speculation that anti-gp39 mediates its immunosuppressive effects by blocking gp39 binding to CD40 and not by the inactivation of gp39-bearing T_h. In support of this hypothesis *in vitro* studies have established that anti-gp39 blocks the binding of CD40 to gp39 (16). Furthermore, biologically active anti-gp39 could be detected in serum for the period of time that immune suppression was apparent. Although only 5% of anti-gp39 was present in serum at a time when immune suppression was evident, it is possible that the local tissue concentrations of anti-gp39 in specific sites of secondary lymphoid organs is higher and clearance rates are slower than that of serum anti-gp39. Treatment of mice with anti-gp39 inhibited the primary immune response to SRBC and heterologous Ig >90% for prolonged periods of time. Assuming that anti-gp39 is mediating the inhibition by blocking gp39 function, these data implicate gp39-CD40 interactions as essential in the development of primary immune responses to TD antigens. Immunohistochemical analysis establish that gp39 is induced as a consequence of immunization with TD antigens and may be of functional significance. The *in situ* studies of gp39 expression illustrate that the initial site of gp39-CD40 interactions during primary humoral immune responses is in the peripheral aspects of the periarteriolar lymphoid sheaths (PALS) and around the terminal arterioles (TA) of the spleen. It is at these sites that conjugates between gp39-expressing T_h and antigen-specific B cells were found juxtaposed,

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suggesting that the outer PALS is a major site of T cell-B cell interactions during primary humoral immune responses. Therefore, the PALS may be the site at which anti-gp39 interacts with gp39-expressing T_H cells to ultimately inhibit T-B interaction and subsequent Ig production.

5 Similar to primary responses, the secondary humoral immune response of mice primed to KLH in CFA was also shown to be inhibited by the administration of anti-gp39. Consistent with the reduction of anti-SRBC PFC by anti-gp39, reductions in serum antibodies titers to antigenic challenge were also observed. The serum titers of all anti-KLH Ig isotypes measured (IgM, IgG₁, IgG_{2a}, IgG_{2b}, IgG₃, and IgE) were reduced by the
10 treatment of mice with anti-gp39. The effect of anti-gp39 administration was apparent for at least 14d after secondary challenge with antigen, establishing a persistent immune suppression by anti-gp39. Anti-gp39-mediated immune suppression of secondary responses to KLH is not unique to KLH, since secondary immune responses to heterologous Ig and heterologous erythrocytes were also inhibited by anti-gp39 therapy. The anatomical
15 distribution of gp39-expressing T_H was identical to that observed upon primary immunization, however, the frequency of gp39-expressing T_H in immune spleen was increased over that observed during primary immune responses. No gp39-expressing T_H were found in the germinal centers or follicles of immune spleen. Thus, it appears that B cells are triggered to respond to activated T_H cells in the PALS and TA of the spleen and later
20 migrate to the follicles and germinal centers.

 The focus of the present study was to demonstrate the potential use of anti-gp39 in the control of TD humoral immunity. Brief treatment regimes with anti-gp39 resulted in prolonged suppression, an attractive attribute of this therapeutic antibody. Of special interest may be the capacity of anti-gp39 to prevent primary and secondary humoral responses to
25 other heterologous, therapeutic antibodies such as Chi-L6. This would permit the exposure of patients to repeated administrations of heterologous therapeutic antibodies.

EXAMPLE 7: Production and Characterization of Anti-gp39 Antibodies

30 Experiment 1 - Antibodies directed against human gp39

 For induction of antigen-specific T cell tolerance in a human subject, it is preferable to administer an antibody directed against human gp39. The following methodology was used to produce mouse anti-human gp39 monoclonal antibodies. Balb/c mice were immunized with a soluble gp39 fusion protein, gp39-CD8, in Complete Freund's Adjuvant
35 (CFA). Mice were subsequently challenged 6 weeks later with soluble gp39-CD8 in Incomplete Freund's Adjuvant (IFA). Soluble gp39-CD8 was given in soluble form 4 weeks after secondary immunization. Mice were then boosted with activated human peripheral blood lymphocytes 2 weeks later, followed by a final boost with soluble gp39-CD8 after an

additional 2 weeks. Splenocytes were fused with the NS-1 fusion partner on day 4 after final immunization as per standard protocols.

Clones producing anti-human gp39 antibodies were selected based on a multiple screening process. Clones were initially screened by a plate binding assay using gp39-CD8. Positive clones were then screened against a control CD8 fusion protein, CD72-CD8. Clones which scored positive on the CD8-CD72 plate binding assay were eliminated. The remaining clones were subsequently screened on resting and 6 hour activated human peripheral blood lymphocytes (PBL) by flow cytometric analysis. Hybridomas staining activated, but not resting, PBL were considered positive. Finally, the remaining clones were tested for their ability to block the binding of CD40Ig to plate bound gp39.

Approximately 300 clones were initially screened against gp39-CD8 and CD72-CD8 in the plate binding assays. Of those clones, 30 were found to detect plate-bound gp39 and not CD8. These clones were subsequently screened for detection of gp39 on activated human PBL. Approximately 15 clones detected a molecule on activated PBL, but not resting cells. Specificity was further confirmed by determining the capacity of the clones to block CD40Ig detection of plate-bound gp39. 3 of 10 clones tested block CD40Ig binding in this assay. These clones were 3E4, 2H5 and 2H8. Such clones are preferred for use in the methods described herein. Clones which tested positive on activated, but not resting PBL, were also screened for reactivity with an activated rat T cell clone, POMC8. The clone 2H8 expressed crossreactivity with this rat T cell line.

Experiment 2 - Antibodies directed against human gp39

A similar immunization procedure to that described in Experiment 1 was used to produce additional antibodies directed against human gp39. One Balb/c mouse was immunized with soluble gp39-CD8 in CFA, followed by challenge with 6 hour activated human peripheral blood lymphocytes 4 weeks later. The mouse was subsequently boosted with soluble gp39-CD8 4 days prior to fusion of splenocytes with the NS-1 fusion partner per standard protocols. Screening of hybridoma clones was performed by flow cytometric staining of 6 hour activated human PBLs. Clones staining activated but not resting human PBLs were selected. Six clones, 4D9-8, 4D9-9, 24-31, 24-43, 89-76 and 89-79, were selected for further analysis.

The specificity of the selected antibodies was confirmed by several assays. First, flow cytometric analysis demonstrated that all six mAbs stain activated, but not resting peripheral blood T cells (see Figure 7B and 7C for a representative example, depicting staining of activated T cells with 4D9-8 and 4D9-9, respectively). Expression of the molecule recognized by each of the six antibodies is detectable within 4 hours of activation, is maximal between 6-8 hours after activation, and is undetectable by 24 hours after activation. All six mAbs recognize a molecule expressed on activated CD3⁺ PBLs, predominantly of the CD4⁺ phenotype, but a portion of CD8⁺ T cells also express the molecule. Expression of the

molecule recognized by the six mAbs is inhibited by the presence of cyclosporin A in the culture medium, as is the expression of gp39 (see Figure 8A and 8B for a representative example, depicting staining of cyclosporin treated T cells with 4D9-8 and 4D9-9, respectively). The kinetics and distribution of expression of the molecule recognized by these mAbs are identical to that of gp39, as detected by the fusion protein of human CD40Ig. In addition, all six mAbs block the staining of gp39 by CD40Ig (see Figure 9A and 9B for a representative example, depicting inhibition of gp39 staining by CD40Ig in the presence of 4D9-8 and 4D9-9, respectively). In an ELISA assay, all six mAbs recognize gp39-CD8, a soluble fusion form of the gp39 molecule. Moreover, all six mAbs immunoprecipitate a molecule of approximately 36 kd from ³⁵S-methionine labeled activated human PBLs. The immunoprecipitated molecule is identical to that precipitated by the human CD40Ig fusion protein.

The functional activity of the six selected mAbs (4D9-8, 4D9-9, 24-32, 24-43, 89-76 and 89-79) was assayed as follows. First, the ability of the mAbs to inhibit the proliferation of purified human B cells cultured with IL-4 and soluble gp39 was measured. Purified human B cells were cultured with gp39 and IL-4 in the presence or absence of purified monoclonal antibodies or CD40Ig at dosages between 0 and 12.5 µg/ml. B cell proliferation was determined after 3 days in culture by thymidine incorporation. The results (shown in Figure 10) demonstrate that all six mAbs can inhibit B cell proliferation induced by gp39 and IL-4. The mAbs 89-76 and 24-31 were most effective at inhibiting the induced B cell proliferation.

Next, the ability of the mAbs to inhibit B cell differentiation, as measured by Ig production induced by anti-CD3 activated T cells and IL-2, was examined. Purified IgD⁺ human B cells were prepared by positive selection with FACS and then cultured with anti-CD3 activated human T cells (mitomycin C treated) and IL-2 for 6 days in the presence or absence of purified anti-gp39 monoclonal antibodies as dosages between 0 and 10 µg/ml. IgM, IgG and IgA production was assessed by ELISA on day 6. The results (shown below in Table 1) demonstrate that all six antibodies can inhibit T cell dependent B cell differentiation, as measured by IgM, IgG and IgA production.

Table 1

mAb	$\mu\text{g/ml}$	Production of Immunoglobulin		
		IgM	IgG	IgA
none	-	17,500	6710	4471
4D9-8	0.1	4813	2130	2819
	1.0	4394	2558	1519
	10.0	1081	389	396
4D9-9	0.1	3594	919	1731
	1.0	2659	1233	1606
	10.0	374	448	266
24-31	0.1	3863	981	344
	1.0	1287	314	165
	10.0	1120	596	23
24-43	0.1	6227	4132	432
	1.0	3193	2130	192
	10.0	7021	1232	1081
89-76	0.1	3783	1069	344
	1.0	2180	352	171
	10.0	818	551	19
89-79	0.1	9763	1924	3021
	1.0	2314	460	156
	10.0	183	135	434

- 5 To examine the effect of the anti-gp39 mAbs on T cell responses, the mAbs were included in standard mixed lymphocyte reactions (MLR). 300,000 human peripheral blood lymphocytes (responders = R) were cultured with 100,000 irradiated allogeneic peripheral blood lymphocytes (stimulators = S) in the presence or absence of anti-gp39 mAbs (10 $\mu\text{g/ml}$). Cultures were pulsed with 3H-thymidine on day 4, 5 or 6 and harvested 18 hours
- 10 later. All six anti-human gp39 mAbs inhibited allo-specific responses as measured by MLR (see Figure 11 for a representative example, depicting inhibition of allo-specific responses when R and S are incubated in the presence of 24-31 or 89-76; a CTLA4-immunoglobulin fusion protein and an anti-CD28 mAb were used as positive controls).

- 15 To determine whether the six mAbs recognized distinct epitopes on the human gp39 molecule, crossblocking experiments were performed. Activated human PBLs were first blocked with each of the six mAbs (25 $\mu\text{g/ml}$ of unconjugated antibody). Cells were washed and then stained with 10 $\mu\text{g/ml}$ of biotin-conjugated antibody, followed by reaction with

phytoerythrin conjugated avidin (PE-Av). The staining of the cells with PE-Av was analyzed by FACS. The results are shown below in Table 2.

Table 2

Blocking Ab	Staining Antibody					
	4D9-8	4D9-9	24-31	24-43	89-76	89-79
none	+++	+++	++++	++++	++++	++++
4D9-8	ND	-	++++	++++	+++	+++
4D9-9	+++	ND	+++	++++	+++	+++
24-31	+	+	ND	+++	++	++
24-43	+	+	+++	ND	++	+
89-76	+	+	+++	+++	ND	+++
89-79	+	++	+++	+++	+++	ND

5

The intensity of staining and the percentage of positive cells are represented by the + symbol (++++ = MI >200; +++ = MI >125; ++ = MI >50; + = MI >25; - = no staining above background). ND= not determined.

- 10 All antibodies blocked the binding of CD40Ig to activated human PBLs. However, the data shown in Table 2 clearly demonstrate the failure of some antibodies to block the binding of other antibodies to activated human PBLs, suggesting that they recognize distinct epitopes on the human gp39 molecules.

- 15 The 89-76 and 24-31 hybridomas, producing the 89-76 and 24-31 antibodies, respectively, were deposited under the provisions of the Budapest Treaty with the American Type Culture Collection, Parklawn Drive, Rockville, Md., on September 2, 1994. The 89-76 hybridoma was assigned ATCC Accession Number _____ and the 24-31 hybridoma was assigned ATCC Accession Number _____.

20 Experiment 3- Antibodies directed against mouse gp39

In one embodiment of the invention, the gp39 antagonist is an anti-mouse gp39 monoclonal antibody, MR1. The following method was used to produce the MR1 monoclonal antibody, and may be used to generate other antibodies directed toward gp39.

- 25 Hamsters were immunized intraperitoneally with $5 \cdot 10^6$ activated T_H1 cells (d1.6) at weekly intervals for six weeks. When the serum titer against murine T_H1 was greater than about 1:10,000, cell fusions were performed with polyethylene glycol using immune hamster splenocytes and NS-1. Supernatant from wells containing growing hybridomas were screened by flow cytometry on resting and activated T_H1 . One particular hybridoma, which produced a Mab that selectively recognized activated T_H1 was further tested and subcloned to
- 30 derive MR1. MR1 was produced in ascites and purified by ion exchange HPLC. A

hybridoma MR1 has been deposited with the American Type Culture Collection and assigned Accession Number HB11048.

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10 **Equivalents**

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

CLAIMS

- 5 1. A method for inhibiting a humoral immune response to a TD antigen *in vivo* comprising administering to a subject the TD antigen with an antagonist of a molecule on a Th cell which mediates contact-dependent helper effector function.
2. The method of claim 1 wherein the molecule on a Th cell which mediates contact-
10 dependent helper effector function is gp39.
3. The method of claim 2 wherein the antagonist is an anti-gp39 antibody.
4. A method for inhibiting a humoral immune response to a TD antigen *in vivo*
15 comprising administering to a subject the TD antigen with a gp39 antagonist.
5. The method of claim 4 wherein the humoral immune response is a primary humoral immune response.
- 20 6. The method of claim 4 wherein the humoral immune response is a secondary humoral immune response.
7. The method of claim 4 wherein the TD antigen is a protein.
- 25 8. The method of claim 4 wherein the TD antigen is an antibody.
9. The method of claim 4 wherein the TD antigen is on a cell surface.
10. The method of claim 4 wherein the gp39 antagonist is an anti-gp39 antibody.
30
11. The method of claim 10, wherein the anti-gp39 antibody is a monoclonal antibody 24-31 (ATCC Accession No. _____).
12. The method of claim 10, wherein the anti-gp39 antibody is a monoclonal antibody
35 89-76 (ATCC Accession No. _____).
13. The method of claim 10 wherein the anti-gp39 antibody is a chimeric monoclonal antibody.

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14. The method of claim 10 wherein the anti-gp39 antibody is a humanized monoclonal antibody.
15. The method of claim 4 wherein the gp39 antagonist is a soluble form of a gp39 ligand.
16. The method of claim 15 wherein the gp39 ligand is CD40.
17. The method of claim 16 wherein CD40 is a fusion protein.
18. The method of claim 4 wherein humoral immune responses to TI-2 antigens are not inhibited.
19. A method for inhibiting an antigen specific IgE response to a TD antigen *in vivo* comprising administering to a subject exposed to the TD antigen a gp39 antagonist.
20. The method of claim 19 wherein the gp39 antagonist is an anti-gp39 antibody.
21. The method of claim 20 wherein the anti-gp39 antibody is a monoclonal antibody 24-31 (ATCC Accession No. _____).
22. The method of claim 20, wherein the anti-gp39 antibody is a monoclonal antibody 89-76 (ATCC Accession No. _____).
23. The method of claim 20 wherein the anti-gp39 antibody is a chimeric monoclonal antibody.
24. The method of claim 20 wherein the anti-gp39 antibody is a humanized monoclonal antibody.
25. The method of claim 19 wherein the gp39 antagonist is a soluble form of a gp39 ligand.
26. The method of claim 25 wherein the gp39 ligand is CD40.
27. The method of claim 26 wherein CD40 is a fusion protein.
28. A method for inhibiting a humoral immune response to a therapeutic agent *in vivo* comprising administering to a subject the therapeutic agent with a gp39 antagonist.

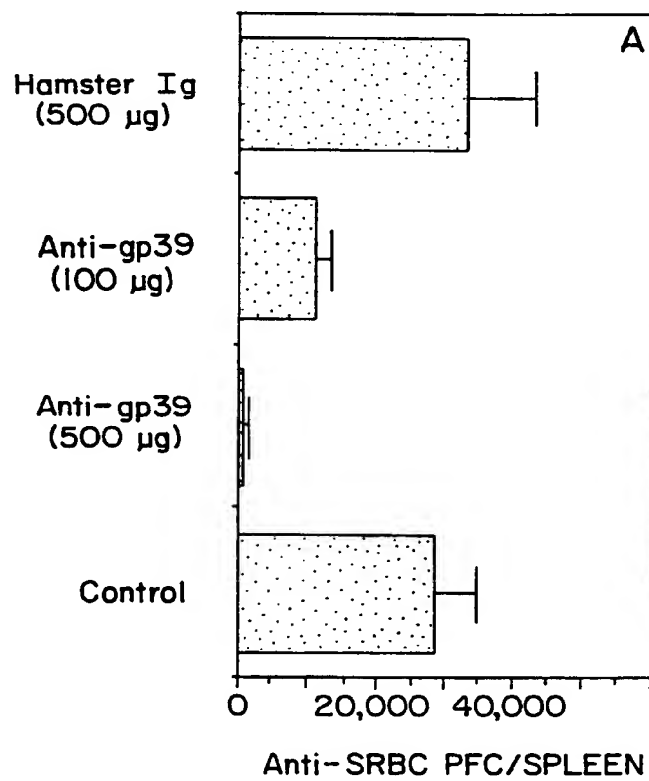
29. The method of claim 28 wherein the therapeutic agent is an antibody.
30. The method of claim 28 wherein the gp39 antagonist is an anti-gp39 antibody.
- 5 31. The method of claim 30 wherein the anti-gp39 antibody is a monoclonal antibody 24-31 (ATCC Accession No. _____).
32. The method of claim 30, wherein the anti-gp39 antibody is a monoclonal antibody
10 89-76 (ATCC Accession No. _____).
33. The method of claim 30 wherein the anti-gp39 antibody is a chimeric monoclonal antibody.
- 15 34. The method of claim 30 wherein the anti-gp39 antibody is a humanized monoclonal antibody.
35. The method of claim 28 wherein the gp39 antagonist is a soluble form of a gp39
ligand.
20
36. The method of claim 35 wherein the gp39 ligand is CD40.
37. The method of claim 36 wherein CD40 is a fusion protein.
- 25 38. A method for inhibiting a humoral immune response to an allergan *in vivo* comprising administering to a subject exposed to the allergan a gp39 antagonist.
39. The method of claim 38 further comprising administering an IL-4 inhibitor to the
subject.
30
40. The method of claim 39 wherein the IL-4 inhibitor is an anti-IL-4 antibody.
41. A method for prolonged suppression of a humoral immune response to a TD antigen
in vivo comprising administering to a subject the TD antigen with a gp39 antagonist.
35
42. A method for inhibiting a humoral immune response to a TD antigen *in vivo* while preserving humoral immune responses to TI-2 antigens comprising administering to a subject the TD antigen with a gp39 antagonist.

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43. A method for immunosuppressing function of activated Th cells induced by a TD antigen *in vivo* comprising administering to a subject the TD antigen with a gp39 antagonist.
44. The method of claim 43 wherein activated Th cells are not deleted.
- 5 45. The method of claim 43 wherein activated Th cells are not anergized.
46. A method for determining whether an antigen is a TD antigen or a TI-2 antigen, comprising:
- 10 (a) administering to a subject the antigen to be tested with a gp39 antagonist;
- (b) measuring humoral immune responses against the antigen; and
- 15 (c) determining that the antigen is a TD antigen by an absence of humoral immune responses or determining that the antigen is a TI-2 antigen by a presence of humoral immune responses.

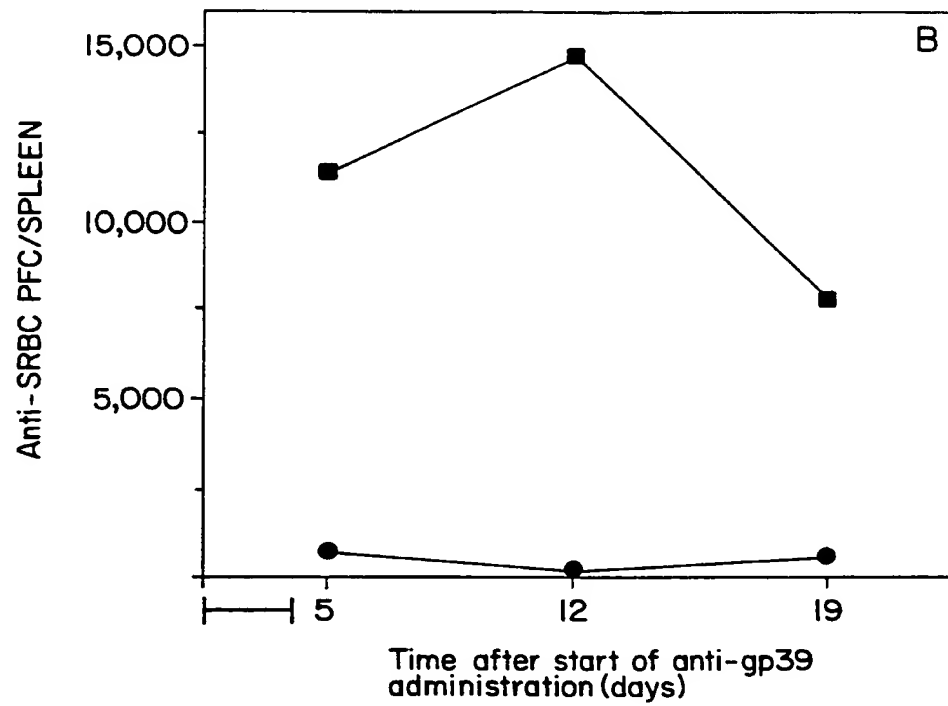
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FIG. 1A



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FIG. 1B



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FIG. 2A

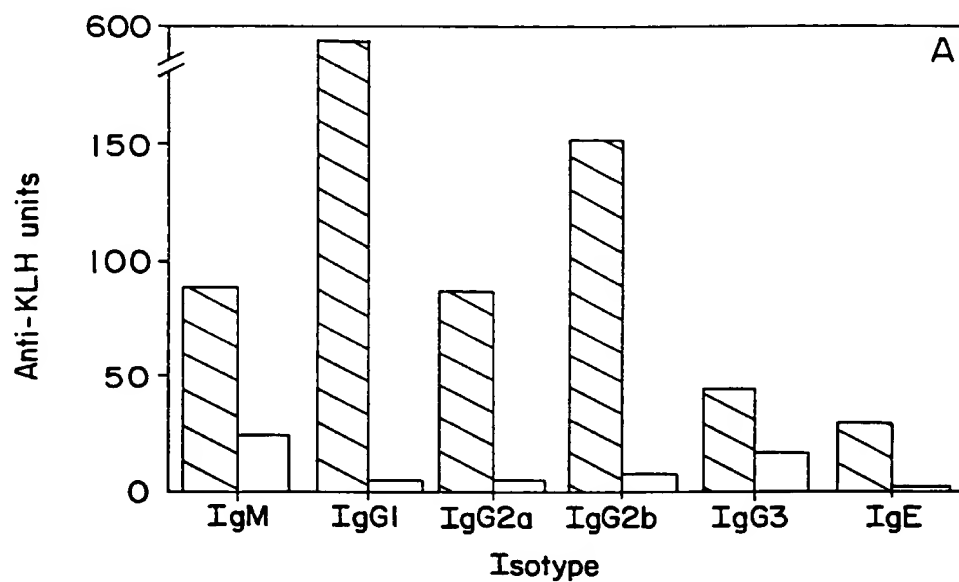
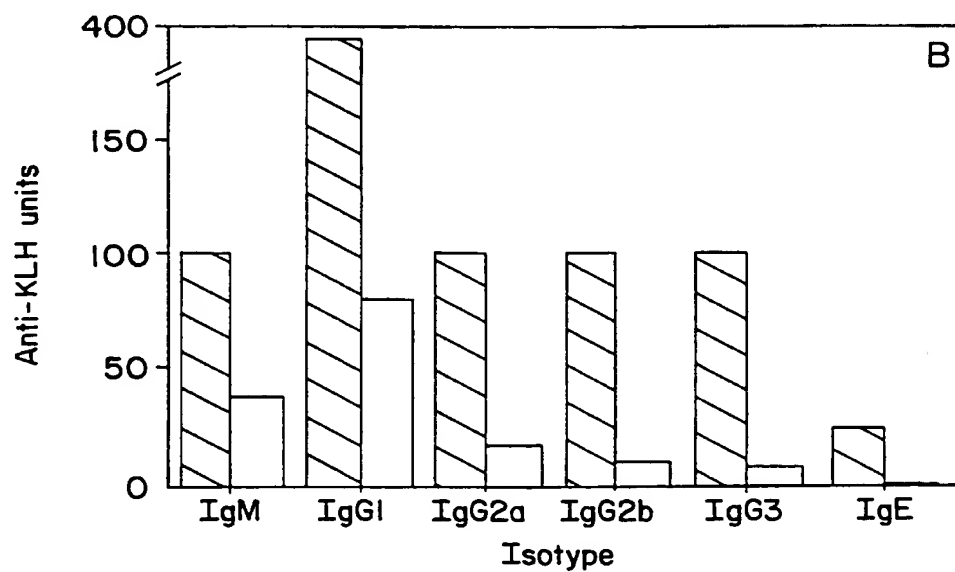


FIG. 2B



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FIG. 3A

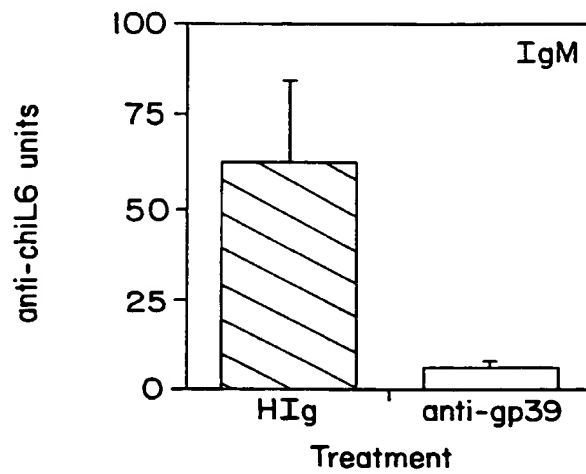
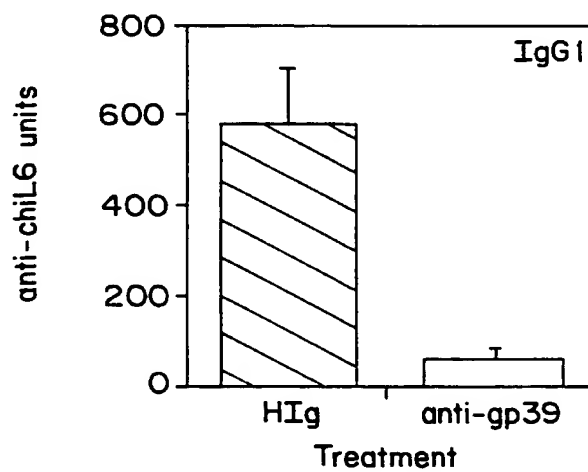


FIG. 3B



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FIG. 4A

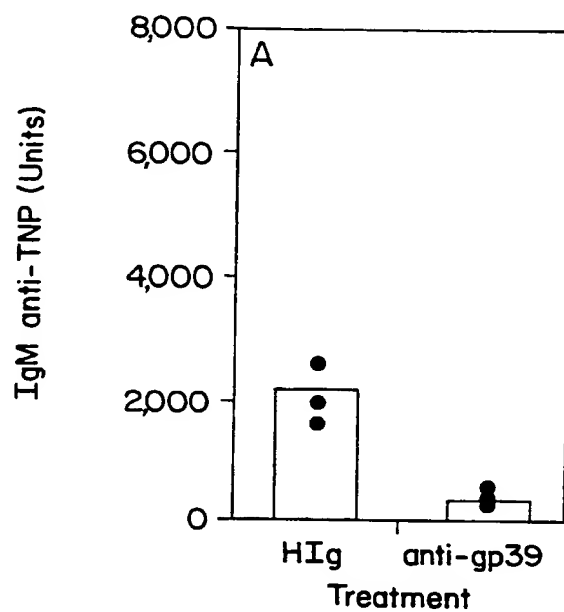
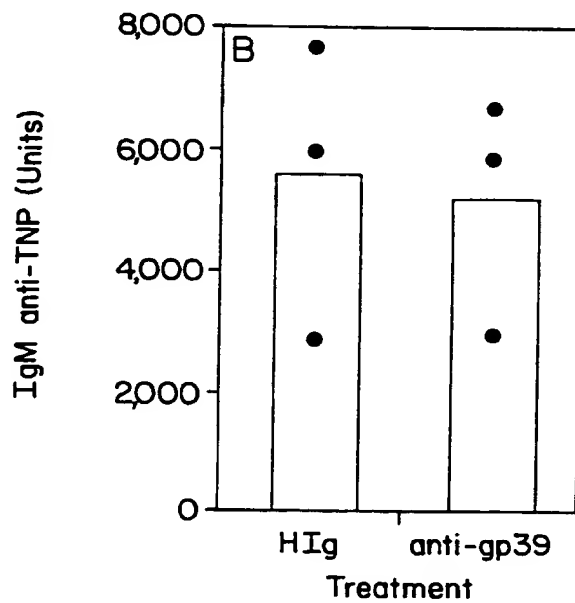
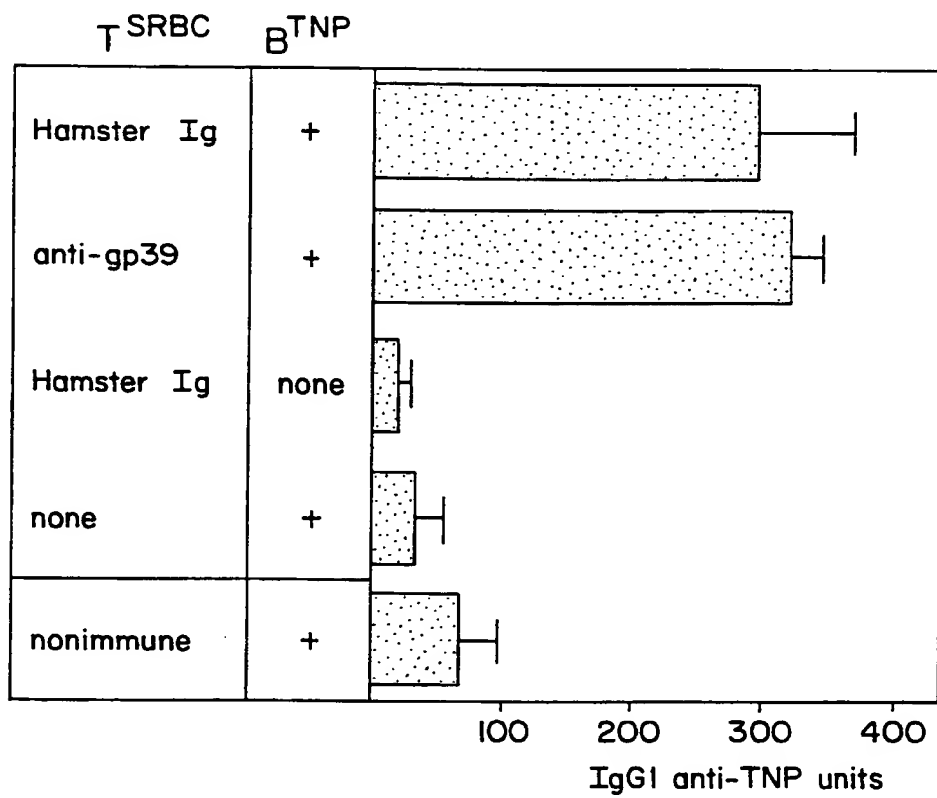


FIG. 4B



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FIG. 5



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FIG. 6A

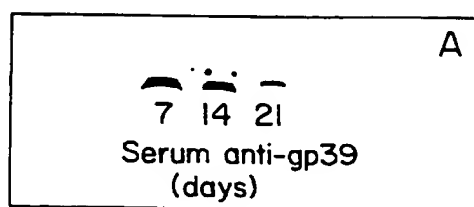
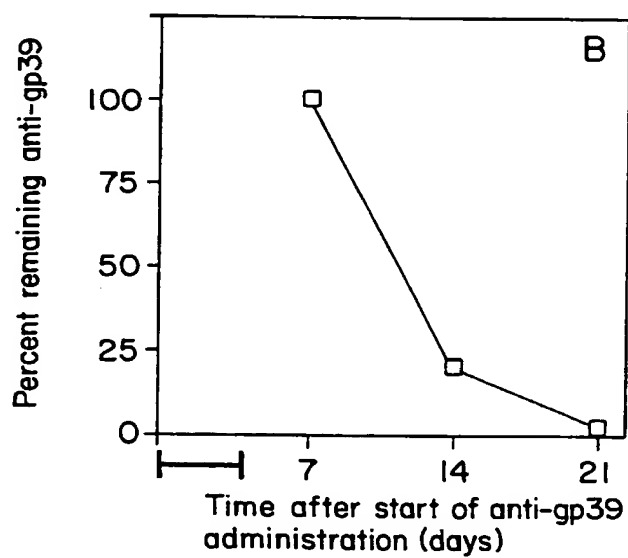


FIG. 6B



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FIG. 7A

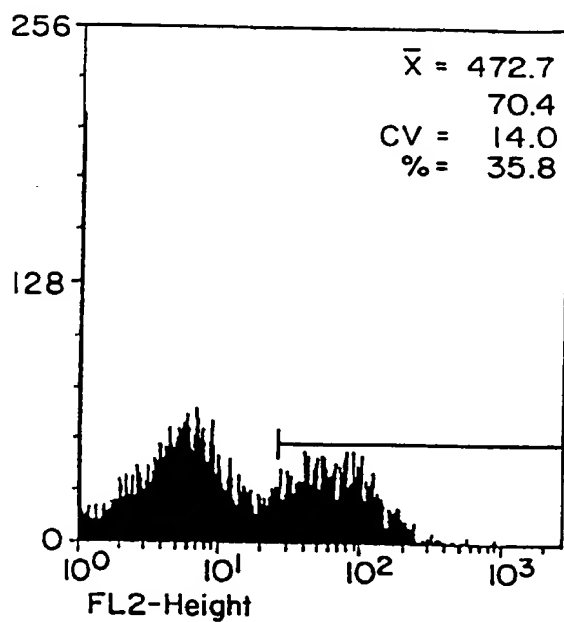
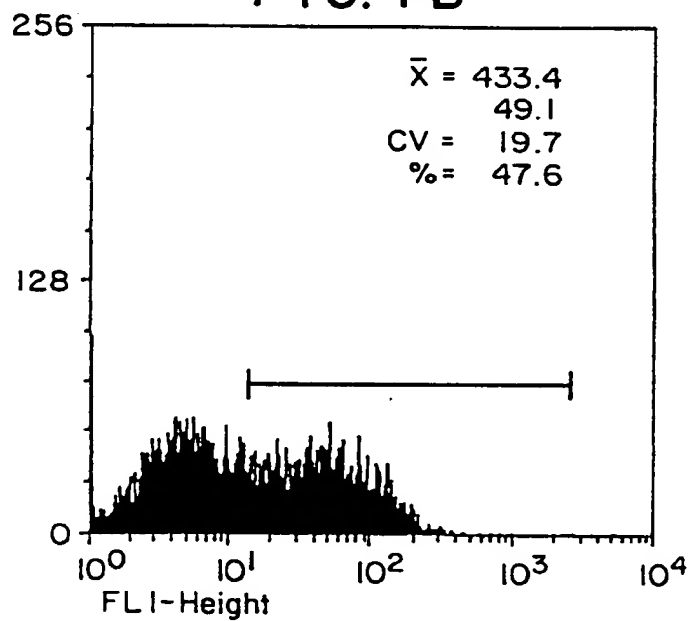


FIG. 7B



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FIG. 7C

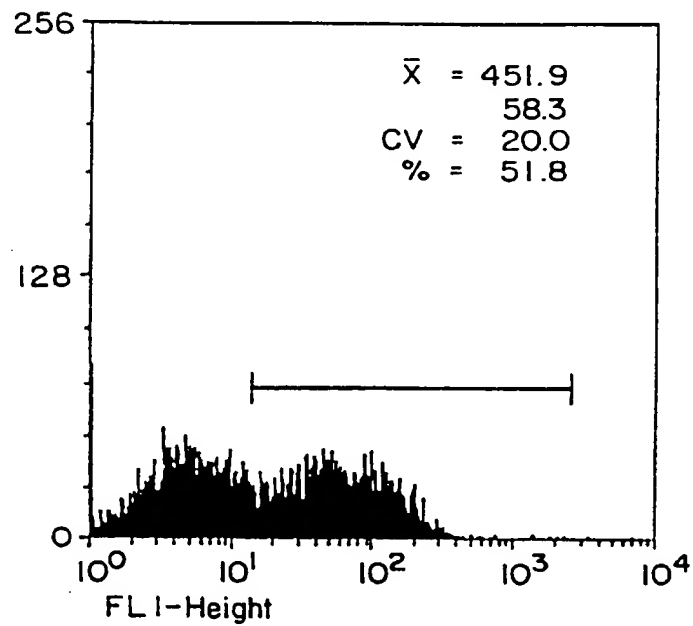
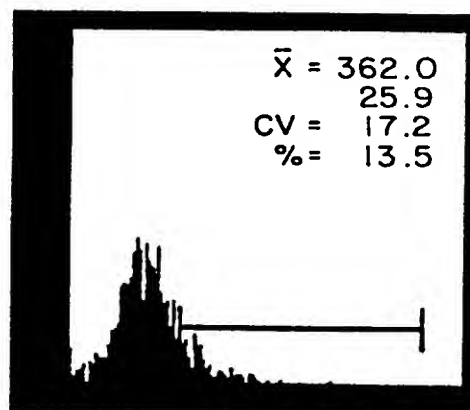


FIG. 8A



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FIG. 8B

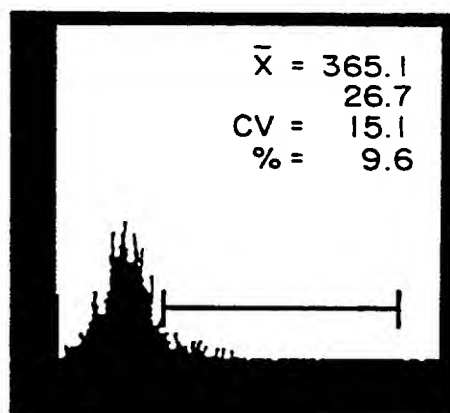
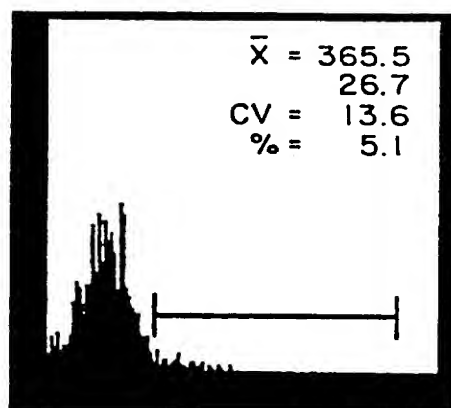


FIG. 8C



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FIG. 9A

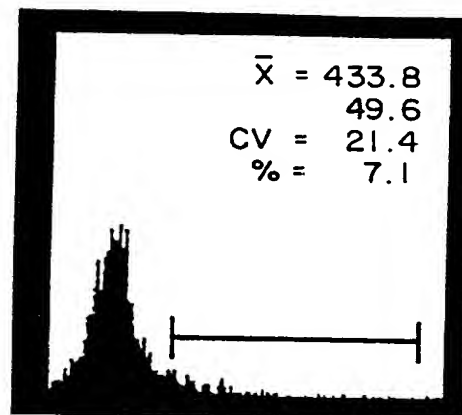
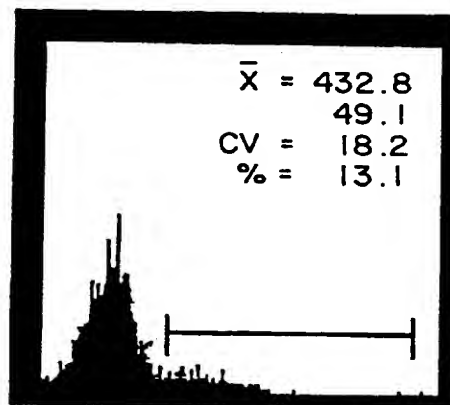


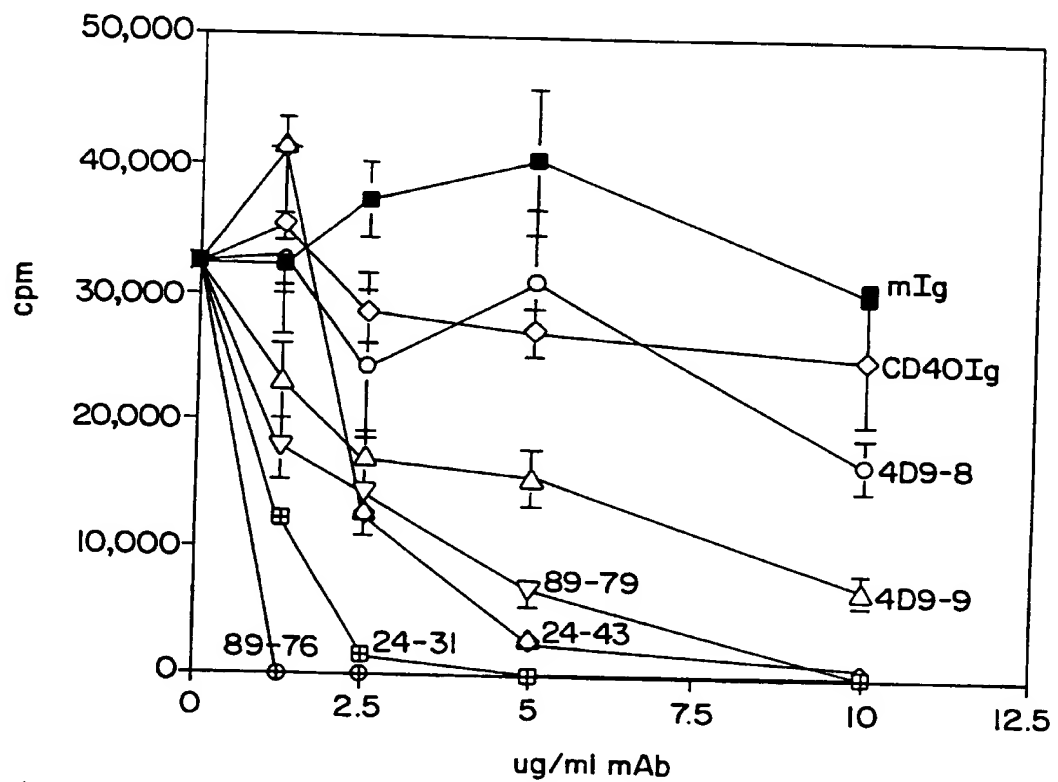
FIG. 9B



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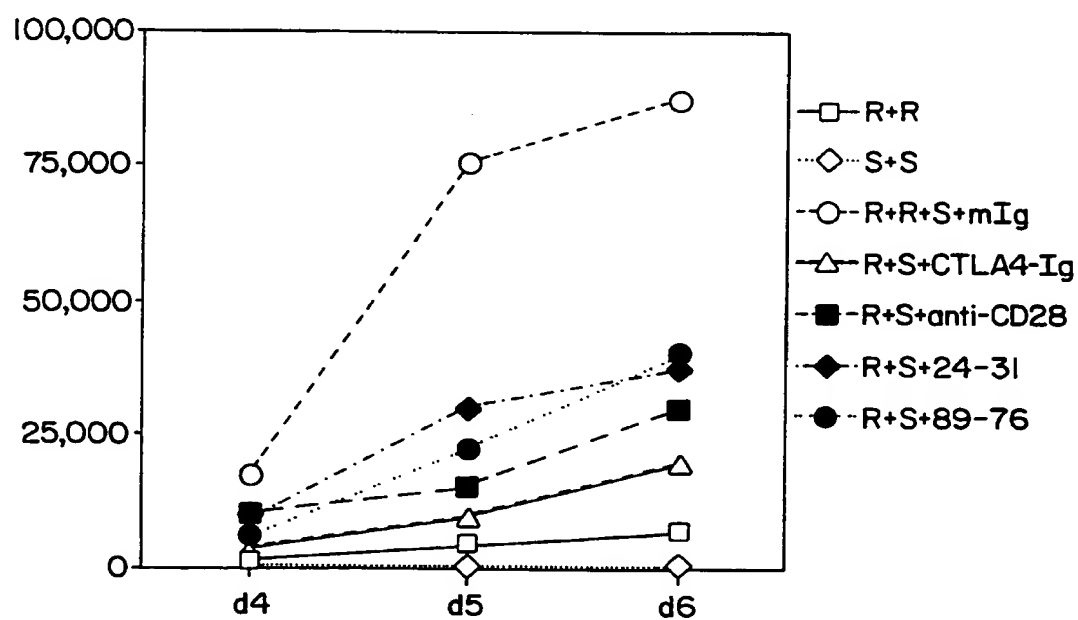
FIG. 10



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FIG. II



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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 94/09872

A. CLASSIFICATION OF SUBJECT MATTER IPC 6 A61K39/395 A61K38/17 G01N33/68 //C12P21/08		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) IPC 6 C07K G01N		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP,A,0 555 880 (BRISTOL-MYERS SQUIBB COMPANY ET AL.) 18 August 1993 see column 14, line 4 - column 20, line 41 see claims ---	1-8,10, 13-18, 28-30, 33-37, 41-46
X	WO,A,93 08207 (IMMUNEX CORPORATION) 29 April 1993 see examples 7,8,12,13 see claims 15,18,19 --- -/--	1,2,4-6, 15,19, 25-28, 35-38, 41,43-45
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "B" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. "&" document member of the same patent family		
Date of the actual completion of the international search 14 December 1994		Date of mailing of the international search report 28.12.94
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016		Authorized officer Nooij, F

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 94/09872

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>JOURNAL OF CLINICAL IMMUNOLOGY, vol.13, no.3, May 1993, NEW YORK NY, USA pages 165 - 174 L. MARSHALL ET AL. 'The molecular basis for T cell help in humoral immunity: CD40 and its ligand, gp39.' see page 167, right column, line 17 - line 28 see page 168, right column, line 49 - page 169, left column, line 38 see page 172, left column, line 10 - line 30</p> <p>---</p>	<p>1-10, 13-18, 28-30, 33-38, 41-45</p>
X	<p>IMMUNOLOGY TODAY, vol.13, no.11, November 1992, AMSTERDAM, THE NETHERLANDS pages 431 - 433 R. NOELLE ET AL. 'CD40 and its ligand, an essential ligand-receptor pair for thymus-dependent B-cell activation.' see page 432, left column, line 6 - line 20 see page 433, left column, line 4 - line 21</p> <p>---</p>	<p>1-10,13, 14,18, 28-30, 33,34, 41-45</p>
A	<p>PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol.89, no.14, 15 July 1992, WASHINGTON DC, USA pages 6550 - 6554 R. NOELLE ET AL. 'A 39-kDa protein on activated helper T cells binds CD40 and transduces the signal for cognate activation of B cells.' cited in the application see the whole document</p> <p>---</p>	<p>1-10, 13-18, 28-30, 33-37, 41-45</p>
A	<p>THE JOURNAL OF IMMUNOLOGY, vol.149, no.2, 15 July 1992, BALTIMORE MD, USA pages 655 - 660 W. FANSLOW ET AL. 'Soluble forms of CD40 inhibit biologic responses of human B cells.' see abstract see page 657, right column, line 6 - page 658, right column, line 2 see page 658, right column, line 30 - page 659, left column, line 7 see table I</p> <p>---</p>	<p>1,2,4-6, 15-19, 25-28, 35-38, 41-45</p>

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 94/09872

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	THE JOURNAL OF EXPERIMENTAL MEDICINE, vol.178, no.5, 1 November 1993, NEW YORK NY, USA pages 1567 - 1575 T. FOY ET AL. 'In vivo CD40-gp39 interactions are essential for thymus-dependent humoral immunity. II. Prolonged suppression of the humoral immune response by an antibody to the ligand for CD40, gp39.' see abstract see discussion ----	1-10,13, 14, 18-20, 23,24, 28-30, 33,34, 41-45
P,X	THE JOURNAL OF EXPERIMENTAL MEDICINE, vol.180, no.1, 1 July 1994, NEW YORK NY, USA pages 157 - 163 T. FOY ET AL. 'gp39-CD40 interactions are essential for germinal center formation and the development of B cell memory.' see abstract -----	1-7,9, 10,13, 14, 28-30, 33,34, 41-45

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 94/ 09872

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

See Annex
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

Remark : Although claims 1-45 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

Remark : Although claim 46 is directed to a diagnostic method practised on the human/animal body, the search has been carried out on the alleged effects of the compound/composition.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/US 94/09872

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A-0555880	18-08-93	AU-A-	3298893	19-08-93
		CA-A-	2089229	15-08-93
		JP-A-	6220096	09-08-94

WO-A-9308207	29-04-93	AU-A-	3122693	21-05-93
		CA-A-	2121798	29-04-93
		FI-A-	941837	30-05-94
